

Amendments to the Specification:

Please insert the following paragraph on page 1, line 4:

This application is a continuation of U.S. Application No. 10/105,319, filed March 26, 2003, the entire disclosure of which is incorporated herein by reference in its entirety.

Please replace the paragraph starting on page 1, line 21 with the following amended paragraph:

It has been reported that a natural enzyme, deacetoxycephalosporin deacetoxycephalosporin C synthase (DACES, or expandase), may be responsible for the catalysis of the expansion reaction. *Streptomyces* sp. (such as *Streptomyces clavuligerus*, *Streptomyces ambofaciens* and *Streptomyces chartreusis*) can produce the expandase. As illustrated in EP-A-0341892, the expandase can be obtained from *Streptomyces clavuligerus*, and has been cloned. The expandase has been well studied for its chemical and functional properties, see EP-A-03663 54. Unfortunately, the native expandase has less substrate specificity to penicillin G than the normal substrate penicillin N (Rollins, M. J. et al., *Can. J Microbiol.* **34**: 1196-1202 (1988) and Crawford, L., et al., *Bio/Technology*, **13**: 58-62 (1995)). Penicillin G is commercially available at a low cost. In contrast, penicillin N is expensive and not easily available. Furthermore, even though penicillin N is expanded, its side chain cannot be easily removed. Accordingly, the chemical synthesis of 7-ADCA, rather than an enzymatical synthesis, is still used in industrial production.

Please replace the paragraph starting on page 3, line 12 with the following amended paragraph:

One object of the invention is to provide a mutated penicillin expandase which comprises an amino acid substitution at one or more residue positions corresponding to those of a wild-type expandase selected from the group consisting of methionine 73, serine glycine 79, valine 275, leucine 277, cysteine 281, glycine 300, asparagine 304 and isoleucine 305, provided that the amino acid substitution at the residue position of asparagine 304 is not N304L. In particular, the invention provides a mutated penicillin expandase which comprises one or more specific amino acid substitutions selected from the group consisting of M73T, S79E G79E, V275I, L277K, C281Y, G300V, N304K, I305L and I305M, wherein the residue positions of the amino acid substitution correspond to those of a wild-type expandase.

Please replace the paragraph starting on page 6, line 2 with the following amended paragraph:

The primary aspect of the present invention is to provide a mutated penicillin expandase having a better substrate specificity to penicillin G, wherein the mutated penicillin expandase comprises an amino acid substitution at one or more residual positions corresponding to those of a wild-type expandase selected from the group consisting of methionine 73, ~~serine~~ glycine 79, valine 275, leucine 277, cysteine 281, glycine 300, asparagine 304 and isoleucine 305, provided that the amino acid substitution at the residue position of asparagine 304 is not N304L. More specifically, the invention provides a mutated penicillin expandase comprising one or more specific amino acid substitutions selected from the group consisting of M73T, ~~S79E~~ G79E, V275I, L277K, C281Y, G300V, N304K, I305L and I305M.

Please replace the paragraph starting on page 6, line 21 with the following amended paragraph:

The mutated penicillin expandase of the invention comprises the functional equivalents of the same. As used herein, the “functional equivalents” of the mutated penicillin expandase may contain further amino acid mutations (e.g., deletions, additions or substitutions) located at positions other than those described above, wherein said further amino acid mutations result in silent changes and thus do not substantially affect the function (e.g., enzyme activity) of the mutated penicillin expandase. Furthermore, in the “functional equivalents” of the mutated penicillin expandase, the specific amino acid substitutions (i.e., selected from M73T, ~~S79E~~ G79E, V275I, L277K, C281Y, G300V, N304K, I305L and I305M) may be exchanged with other amino acid substitutions of similar characteristics which result in a silent change. For example, a mutated penicillin expandase with an amino acid substitution of “~~S79D~~ G79D” is functional equivalent to that with an amino acid substitution of “~~S79E~~ G79E”, since the amino acid D (aspartic acid) and E (glutamic acid) are both classified as acid amino acids and are of similar characteristics.

Please replace the paragraph starting on page 12, line 18 with the following amended paragraph:

The mutated transformants were grown in a 96-well plate containing 56.7 μ l of LB medium with kanamycin in each well. Then 0.1 mM IPTG was added into each well to induce DAOCS indirectly at 30°C for 2 hours of shaking, followed by another 1 hour of shaking after an addition of 7 μ l of 100 mg/ml lysozyme. The activity of DAOCS was measured by an addition of 30 μ l of assay mixture (500 mM Mops/pH 7.0, 18 mM FeSO₄, 40

mM ascorbate, 25.6 mM α -ketoglutarate and suitable amount of penicillin G), and incubated at 30°C for another 1 hour of shaking. The resulting mixture was loaded on an 8 mm thick paper disc, and placed onto a bioassay plate seeded with *Escherichia coli* ESS strain (a β -lactum supersensitive mutant, a gift from Dr. Demain) as described in Cho H. et al., *Proc. Natl. Acad. Sci. USA*, **95**: 11544-11548 (1988). The transformants with clear zone bigger than that of unmutated control strain were selected and subjected for further activity confirmation by TLC. After the activity improvement screening, the mutated *cefE* was manipulated into a NdeI-Hind III insertion version in the same vector (pET24a), and the mutants such as YS5 (V275I), YS53 (C281Y) and YS59 (S79E) (G79E) were selected.

Please replace TABLE 1 on page 16 with the following amended TABLE 1:

Strains	Km (mM)	k_{cat} (S ⁻¹)	k_{cat} / Km (M ⁻¹ S ⁻¹)
<u>For penicillin N:</u>			
YS16 (wild-type)	0.014 ± 0.006	0.307 ± 0.038	22,000
YS5 (V275I)	0.012 ± 0.003	0.252 ± 0.020	20,000
YS8 (I305L)	0.0060.002	0.284 ± 0.030	44,000
YS11 (I305M)	0.012 ± 0.001	0.310 ± 0.004	26,000
YS12 (N304K)	0.004 ± 0.001	0.366 ± 0.023	92,000
YS125 (N304L)	0.018 ± 0.004	0.415 ± 0.063	23,000
YS49 (L277K)	0.011 ± 0.005	0.220 ± 0.042	20,000
YS53 (C281Y)	0.006 ± 0.001	0.273 ± 0.014	47,000
YS59 (<u>S79E</u>) (<u>G79E</u>)	0.009 ± 0.003	0.178 ± 0.019	20,000
YS115 (M73T)	0.006 ± 0.003	0.239 ± 0.009	40,000
YS67 (V275I & I305M)	0.013 ± 0.005	0.316 ± 0.032	24,000
<u>For penicillin G:</u>			
YS16 (wild-type)	2.58 ± 0.22	0.0302 ± 0.0007	12
YS5 (V275I)	1.68 ± 0.20	0.0335 ± 0.0008	20
YS8 (I305L)	0.66 ± 0.07	0.0506 ± 0.0010	77
YS11 (I305M)	0.75 ± 0.04	0.0968 ± 0.0013	129
YS12 (N304K)	0.22 ± 0.03	0.0376 ± 0.0002	171
YS125 (N304L)	0.55 ± 0.12	0.0268 ± 0.0004	49
YS49 (L277K)	0.72 ± 0.02	0.0343 ± 0.0005	48
YS53 (C281Y)	0.68 ± 0.34	0.0496 ± 0.0022	73
YS59 (<u>S79E</u>) (<u>G79E</u>)	0.75 ± 0.02	0.0210 ± 0.0002	28
YS115 (M73T)	0.74 ± 0.16	0.0418 ± 0.0014	56
YS67 (V275I & I305M)	0.25 ± 0.08	0.0972 ± 0.025	389

Please replace the paragraph starting on page 22, line 2 with the following amended paragraph:

A mutated expandase enzyme having higher activity on penicillin G is provided to produce phenylacetyl-7-aminodeacetoxycephalosporanic acid (7-ADCA), which mutated

expandase enzyme has one or more amino acid substitutions selected from M73T, S79E,
G79E, V275I, L277K, C281Y, G300V, N304K, I305L and I305M.